Ctk1 Promotes Dissociation of Basal Transcription Factors from Elongating RNA Polymerase II

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Ctk1 promotes dissociation of basal transcription factors from elongating RNA polymerase II

Seong Hoon Ahn[1,*], Michael-Christopher Keogh[2,3] and Stephen Buratowski[2]

[1]Division of Molecular and Life Science, College of Science and Technology, Hanyang University, Ansan, Republic of Korea and [2]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, USA

As RNA polymerase II (RNApII) transitions from initiation to elongation, Mediator and the basal transcription factors TFIID, TFIIA, TFIIH, and TFIIE remain at the promoter as part of a scaffold complex, whereas TFIIB and TFIIF dissociate. The yeast Ctk1 kinase associates with elongation complexes and phosphorylates serine 2 in the YSPTSPS repeats of the Rpb1 C-terminal domain, a modification that couples transcription to mRNA 3′-end processing. The higher eukaryotic kinase Cdk9 not only performs a similar function, but also functions at the 5′-end of genes in the transition from initiation to elongation. In strains lacking Ctk1, many basal transcription factors cross-link throughout transcribed regions, apparently remaining associated with RNApII until it terminates. Consistent with this observation, preinitiation complexes formed on immobilized templates with transcription extracts lacking Ctk1 leave lower levels of the scaffold complex behind after escape. Taken together, these results suggest that Ctk1 is necessary for the release of RNApII from basal transcription factors. Interestingly, this function of Ctk1 is independent of its kinase activity, suggesting a structural function of the protein.

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Introduction

Transcription by RNA polymerase II (RNApII) requires a large array of proteins to assemble at a basal promoter. An initial committed complex is formed by TFIID binding to the TATA element. This complex exerts an effect as a binding site for TFIIB, which in turn recruits RNApII and TFIIF. TFIIE, TFIIH, and accessory factors, such as Mediator, then associate to complete the preinitiation complex (PIC). Finally, an ATP-dependent activation step is required for transcription to occur (Buratowski, 1994).

Following initiation, different fates are possible for the basal transcription factors. After the synthesis of ~30 bases of RNA, RNApII is thought to break contact with the core promoter and 'escape' into productive elongation (Heintzman and Ren, 2007). Analyses with purified mammalian factors suggested that only TFIID and TFIIA remain at the promoter (Reinberg *et al*, 1987). A more recent *in vitro* study using yeast extracts found that TFIID, TFIIA, TFIIH, TFIIE, and Mediator remain behind at the promoter in a 'scaffold complex' primed for rapid reinitiation (Yudkovsky *et al*, 2000; Hahn, 2004). TFIIB and TFIIF dissociate from the promoter-bound complex, whereas RNApII moves into the elongation phase. At some point thereafter, various elongation and mRNA processing factors join the elongation complex. The signals for these transitions remain unclear, although recent experiments suggest that both Kin28 (TFIIH) and Srb10 (Mediator) kinases are important for scaffold formation (Yudkovsky *et al*, 2000).

Another kinase that has been implicated in early transcription is mammalian Cdk9, the catalytic component of positive transcription elongation factor b (P-TEFb). Originally isolated as a positive elongation factor for *in vitro* transcription (Fu *et al*, 1999), this kinase has been ascribed several functions. Cdk9 is required for an unclarified early event that also involves DSIF and NELF (Wada *et al*, 1998). It is also required for efficient coupling of transcription with mRNA 3′-end processing (Ni *et al*, 2004). The yeast kinases Ctk1 and Bur1 share significant sequence similarity with Cdk9. Chromatin immunoprecipitation (ChIP) experiments show that Ctk1 associates with RNApII throughout elongation (Kim *et al*, 2004a). Similar to Cdk9, Ctk1 couples transcription with polyadenylation by phosphorylating the C-terminal domain (CTD) of Rpb1, the largest subunit of RNApII (Ahn *et al*, 2004). Here, we show that Ctk1 also has an early function in transcription. Basal transcription factors generally localize specifically to promoters, but in cells lacking Ctk1, many are found coincident with RNApII throughout transcriptionally active genes. Furthermore, in extracts from cells lacking Ctk1, much lower levels of scaffold components remain associated with promoters after initiation. These results indicate that Ctk1 may help to trigger the release of basal transcription factors from RNApII as it enters productive elongation.

Results

Ctk1 is necessary for the dissociation of basal transcription factors from elongating RNA polymerase II

We showed previously that Ctk1 phosphorylates serine 2 in the Rpb1 CTD during elongation (Cho *et al*, 2001). This

*Corresponding author. Division of Molecular and Life Science, College of Science and Technology, Hanyang University, 1271 Sa 3-dong, Sangnok-gu, Ansan, Gyeonggi-do 426-791, Republic of Korea.
Tel.: +82 31 400 5518; Fax: +82 31 419 1760;
E-mail: hoon320@hanyang.ac.kr
[3]Present address: Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY 10461, USA

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modification is necessary for efficient recruitment of the Set2 methyltransferase to RNApII within transcribed regions (Krogan *et al*, 2003) and polyadenylation factors at the 3′-end of genes (Ahn *et al*, 2004). However, the majority of elongation factors associate with polymerase independently of Ctk1 (Ahn *et al*, 2004).

To determine whether Ctk1 regulates the disposition of basal transcription factors, ChIP experiments on three constitutively expressed genes (*PMA1*, *ADH1*, and *PYK1*) were performed as described previously (Kim *et al*, 2004a). ChIP analysis using polyclonal antibodies against TBP (TATA-binding protein), TFIIB (Sua7), TFIIE (Tfa2) and TFIIH (Tfb1 and Kin28) generally confirmed the localization of these basal transcription factors at promoter regions in a wild-type (WT) background. Remarkably, in cells lacking Ctk1 (*ctk1*Δ), TBP cross-linked throughout these genes, from the promoter to the 3′-end (Figure 1B). Consistent with this, when ChIP was carried out using strains deleted for the other subunits of the Ctk1 complex, including Ctk2 (cyclin subunit) or Ctk3 (accessory factor), TBP occupancy was again increased in the coding region of *PMA1* (Supplementary Figure S1). This observation was not restricted to TBP, with similar cross-linking patterns observed for TFIIE and TFIIH. Quantitation confirmed that TBP and Kin28 occupancy in the body of actively transcribing genes was significantly higher in *ctk1*Δ compared with WT cells. Spreading of Sua7 (TFIIB), Tfb1 (TFIIH) and Tfa2 (TFIIF) was also observed, although the association of these proteins with promoter regions was decreased in *ctk1*Δ cells (Figure 1C). TFIIB has recently been shown to cross-link with both the terminator and promoter regions of many active genes (Singh and Hampsey, 2007). This association is important for gene looping and dependent on Ssu72, a component of the CPF 3′-end processing complex (Singh and Hampsey, 2007). In accordance with this report, Sua7 cross-linked with region 7, downstream of the *PMA1* poly(A) sites in a WT strain (Figure 1C). This pattern is generally maintained in the absence of Ctk1, suggesting that recruitment of TFIIB to 3′-ends is distinct from that of basal transcription factors to promoters.

To exclude the possibility that the abnormal cross-linking of basal transcription factors in *ctk1*Δ cells was due to issues with the specific antibodies, the experiment was repeated with strains carrying TAP-tagged Tfg1, Tfg2, or Tfg3 (all subunits of TFIIF). ChIP was carried out with IgG-agarose as described previously (Kim *et al*, 2004a). Again, spreading of TFIIF from the promoter throughout the transcribed region was seen in *ctk1*Δ cells (Figure 2, Tfg1–TAP result shown is representative). Some promoter preference was still seen, but the qualitative difference upon Ctk1 deletion was clear.

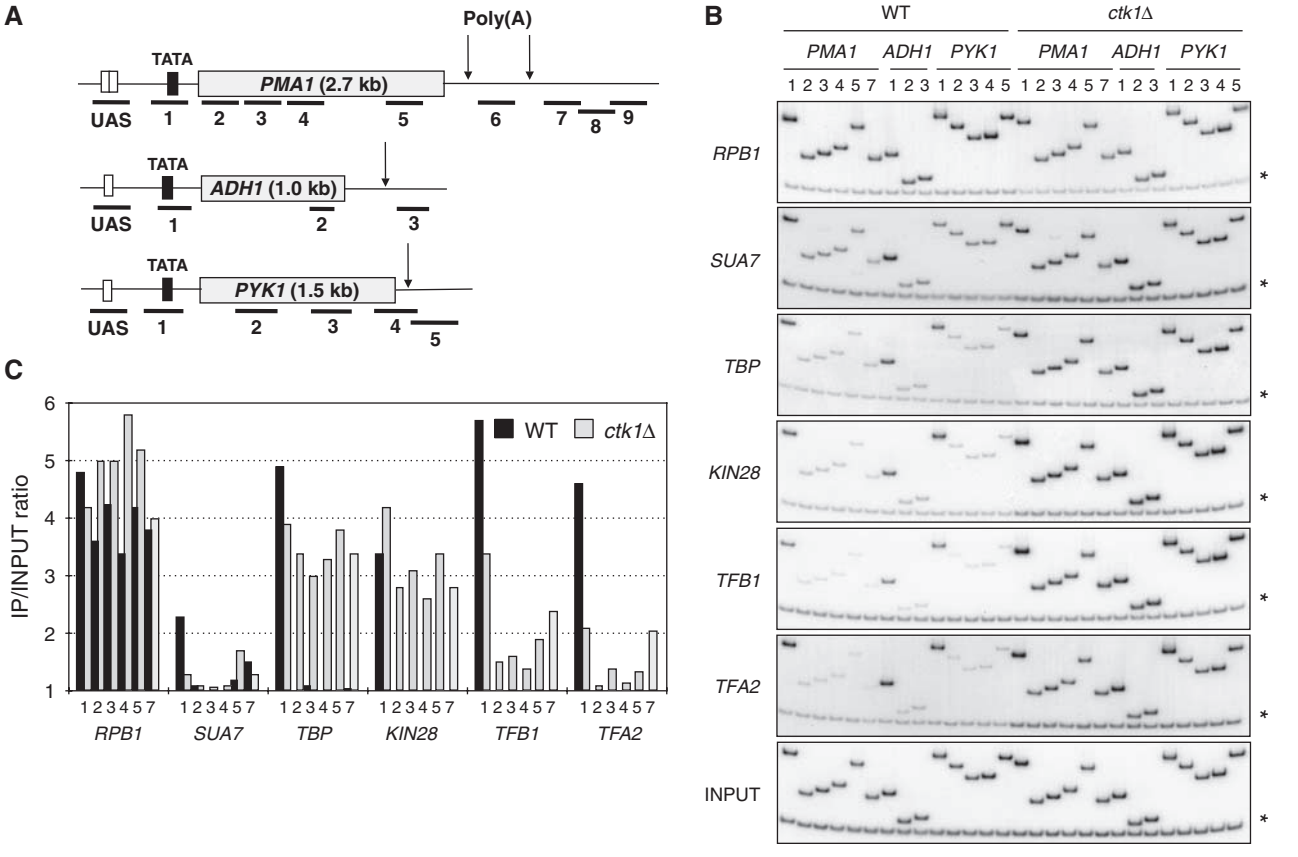

Figure 1 Ctk1 is necessary for the dissociation of basal transcription factors from elongating RNA polymerase II (RNApII). (**A**) Schematic of the *PMA1*, *ADH1*, and *PYK1* genes. The UAS of each gene is indicated by an open box (see Figure 3C). The TATA/promoter region and open reading frames are represented by black and grey boxes, respectively. Arrows indicate the position of the major polyadenylation sites reported previously (Kim *et al*, 2004a) and bars below the genes show the relative positions of PCR products in ChIP analysis. (**B**) Occupancy of Rpb1 and basal transcription factors (Sua7, TBP, Kin28, Tfb1, Tfa2) at the indicated regions in WT (YSB726) or *ctk1*Δ (YSB854) cells. INPUT was used to normalize the PCR amplification and the asterisk marks a non-transcribed PCR fragment indicated in all reactions as a background control. (**C**) Quantitation of the ChIP experiments in (B), with *PMA1* as representative. The *x* axis indicates the specific primer pair used in each PCR. The *y* axis shows the specific signal relative to the negative control (i.e., a ratio of one is equivalent to background).

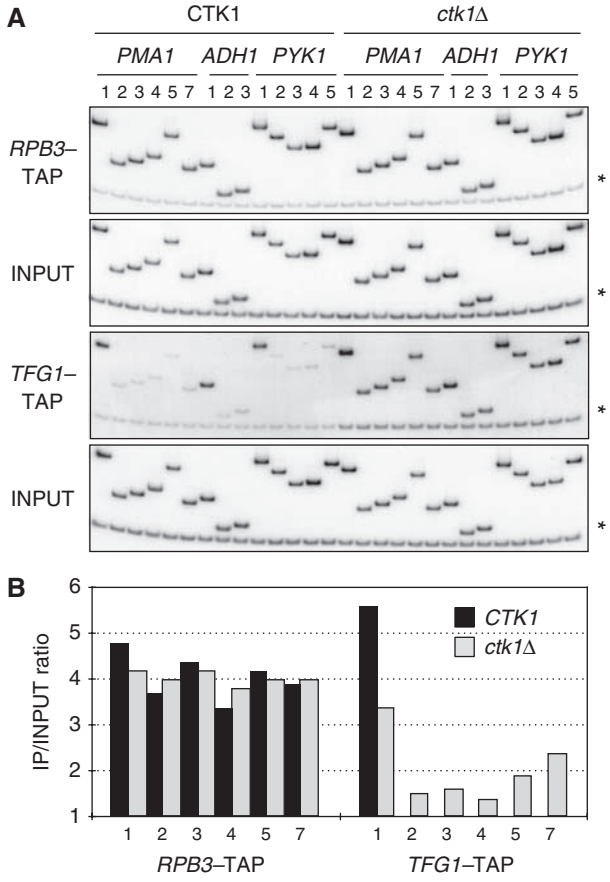

Figure 2 The abnormal cross-linking of basal transcription factors in *ctk1*Δ cells is confirmed by independent immunoprecipitation of TAP-tagged TFIIF subunits. (**A**) ChIP analysis was carried out using Rpb3–TAP (YSB956) or Tfg1–TAP tagged strains (YSB925) +/− Ctk1. Similar cross-linking patterns were seen with Tfg2–TAP (YSB926) or Tfg3–TAP (YSB927) strains (not shown). IgG agarose was used for immunoprecipitation of TAP-tagged proteins. All primer pairs used are described in Figure 1A. PCR products are shown as in Figure 1B. (**B**) Quantitation of results in (A).

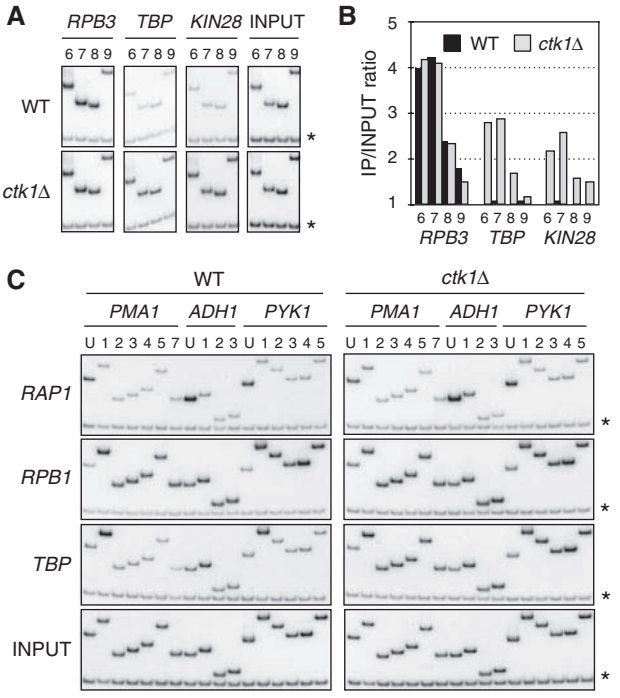

Figure 3 Basal transcription factors coincide with elongating RNApII in *ctk1*Δ cells. (**A**) ChIP analyses were carried out with antibodies against Rpb3, TBP, or Kin28 in WT (YSB726) and *ctk1*Δ (YSB854) backgrounds. Numbers (6–9) correspond to *PMA1* primer locations in Figure 1A. PCR products from (A) and (C) are shown in Figure 1B. (**B**) Quantitation of results in (A). (**C**) Occupancies of Rap1, Rpb1, and TBP at the indicated regions of genes were determined using the indicated polyclonal antibodies in both WT (YSB726) and *ctk1*Δ (YSB854) backgrounds. The upstream activating sequence (UAS) regions of each gene are depicted in Figure 1A.

Several models could account for the mislocalization of basal factors in the *ctk1*Δ background. The first possibility is that transcription initiation complexes are formed at cryptic promoters within gene bodies, a phenotype originally described for mutants in the transcription elongation factor Spt6 (Kaplan *et al*, 2003). Indeed, individual deletion of each member of the Ctk1-complex (Ctk1, Ctk2, or Ctk3) permits cryptic transcription to initiate within *FLO8* (Cheung *et al*, 2008; Fiedler *et al*, 2008). However, several findings argue against this being responsible for the results we observe. First, it is unlikely that every primer pair within the transcribed region of our test genes would contain a cryptic promoter, yet the aberrant cross-linking is relatively even throughout. Second, no additional transcripts were observed when northern blot analysis was performed with 5′- and 3′-probes to *PMA1* (Supplementary Figure S2).

A second model that could explain the cross-linking of basal factors throughout a gene is that they fail to release from RNApII as it moves into productive elongation. To test whether the extended pattern of basal factor cross-linking was completely coincident with RNApII, ChIP experiments were carried out at the termination site for *PMA1* (Figure 3). RNApII levels decreased between primer sets 7 and 9 in WT and *ctk1*Δ strains, indicative of transcription termination in both backgrounds (Ahn *et al*, 2004; Kim *et al*, 2004b). TBP and TFIIH (Kin28) decrease in parallel with RNApII in *ctk1*Δ cells (Figure 3A), supporting the idea that these basal transcription factors travel with elongating RNApII.

We next sought to determine whether the spreading of basal factors is directionally oriented. The upstream activating sequence (UAS; Figure 1A) of the three genes used in our studies (*PMA1*, *ADH1*, and *PYK1*) contain a binding site for repressor-activator protein 1 (Rap 1), a transactivator for many promoters in exponentially growing cells (Rao *et al*, 1993; Lieb *et al*, 2001). The UAS from all three genes show strong Rap1 cross-linking, and this signal remains appropriately localized in *ctk1*Δ cells (Figure 3C). In addition, neither Rpb1 nor TBP are found at the UAS region, indicating that the aberrant cross-linking that we observe in *ctk1*Δ cells is unidirectional along with transcription.

Basal factor spreading does not occur with mutations in other CTD kinases

Three additional cyclin-dependent kinases (Kin28, Srb10, and Bur1) have been reported to phosphorylate the Rpb1 CTD at different stages of a transcription cycle (reviewed by Kobor and Greenblatt, 2002). We tested whether the aberrant localization of basal factors seen with *ctk1*Δ was also observed with mutants in these kinases. The essential Kin28 preferentially phosphorylates S5 of the YSPTSPS repeat, but the *kin28* (T17D) mutant has significantly reduced CTD kinase activity (Keogh *et al*, 2002) that greatly diminishes

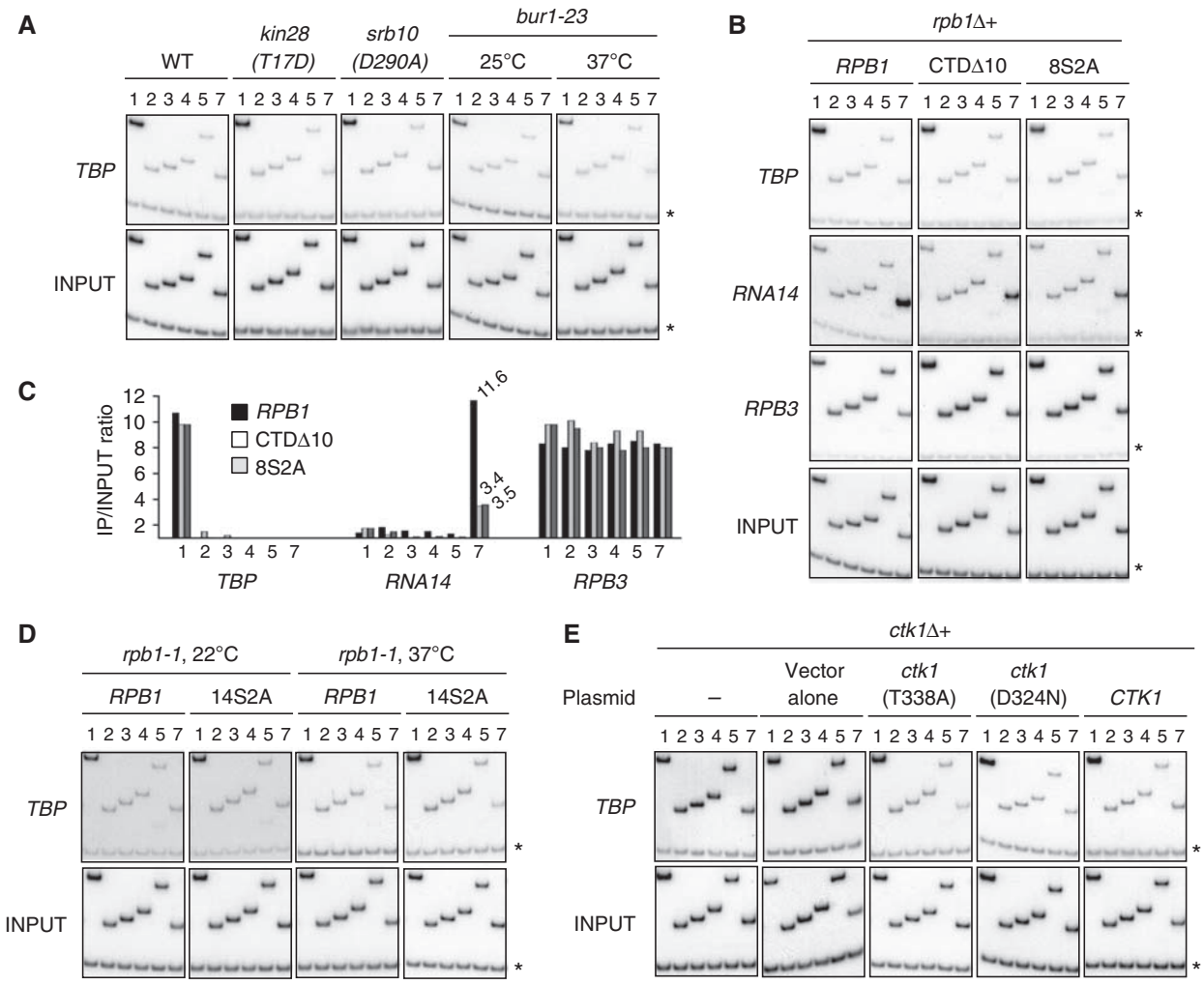

Figure 4 Ctk1 kinase activity is not required for the dissociation of basal transcription factors. (**A**) Three other cyclin-dependent kinases involved in transcription do not regulate the 5′ transitions. Cells with *kin28*-T17D (YSB592) or *srb10*-D290A (YF243) alleles were grown at 30°C and prepared for ChIP analysis. YSB524 is isogenic with YSB592 but contains a WT *KIN28*-covering plasmid and was used as a WT control. The *bur1-23* strain (YSB1021) was grown at 25°C and shifted to 37°C (non-permissive temperature) for 4 h to severely reduce its kinase activity. Chromatin from each strain was immunoprecipitated with anti-TBP. PCR products from (A), (B), (D), and (E) are shown as in Figure 1B. (**B**) Serine 2 phosphorylation of the Rpb1 CTD does not regulate the 5′ transitions. ChIP was carried out in the indicated strains. pRP112 (*RPB1* WT plasmid) in YF68 was replaced by pRP114 (*RPB1*), pY1WT(10) (*rpb1* with 10 WT repeats) or pY1A^2(8)WT(7) (*rpb1* with seven WT and eight S2A repeats). Each strain was then transformed with *RNA14*–TAP (creating YSB1188, YSB1189, and YSB1190, respectively) before ChIP analysis. Protein A- or protein G-sepharose was used for TBP or Rpb3 immunoprecipitation and rabbit IgG agarose was used for *RNA14*–TAP pull-down. (**C**) Quantitation of results from (B). (**D**) A strain with an *rpb1-1* temperature-sensitive allele (YSB784) was transformed with pRP114 (*RPB1* WT copy) or pY1A^2(14) (Rpb1 with 14 S2A mutant repeats of CTD). Cells were grown at 22°C, shifted to 37°C (non-permissive temperature) for 4 h as indicated, and prepared for ChIP analysis. (**E**) The Ctk1-deficient strain (YSB854) was transformed with either of the plasmids YCplac22 (empty vector), YCplac22-CTK1HA (T338A), YCplac22-CTK1-DN, or pRS316-CTK1 and used for ChIP analysis.

capping enzyme recruitment (Rodriguez *et al*, 1999; Komarnitsky *et al*, 2000). Nonessential Srb10 has a preference for S2, and the *srb10* (D290A) mutant is catalytically inactive but incorporated into Mediator (Cho *et al*, 2001). Finally, Bur1 is classed as essential, and the *bur1-23* allele has severely reduced kinase activity that leads to a defect in elongation (Keogh *et al*, 2003). When TBP localization was tested in these mutant backgrounds, no cross-linking was observed outside of the promoter (Figure 4A). This result indicates that the effect on basal transcription factors is specific for Ctk1.

Dissociation of basal factors from RNApII does not require the kinase activity of Ctk1

Ctk1 phosphorylates S2 of the YSPTSPS repeat in the Rpb1 CTD (Cho *et al*, 2001), so this modification is an obvious candidate for mediating basal factor dissociation. The Rpb1 CTD contains 26 copies of the YSPTSPS heptapeptide, and complete deletion of the domain or a mutant in which every serine 2 is replaced by alanine (S2A) is lethal (West and Corden, 1995). However, yeast strains containing a CTD truncated to 10 repeats (CTDΔ10) or with alanine substitutions at half the repeats (seven WT, eight S2A) show conditional growth (West and Corden, 1995). ChIP analysis for TBP was performed in these strains to see whether aberrant cross-linking was observed. As serine 2 phosphorylation is known to be required for cotranscriptional 3′-end processing (Ahn *et al*, 2004), we also tested for the polyadenylation factor Rna14 as a positive control (Figure 4B). In accordance with our previous report, Rna14 cross-linking at the 3′-end of *PMA1* was reduced to about one-third of WT levels. This

was not due to changes in overall RNApII elongation, as monitored by Rpb3. However, TBP did not travel with RNApII in these CTD mutant strains. We also expressed an *RPB1* allele containing 14 repeats of the S2A substitution in the presence of the temperature-sensitive mutant *rpb1-1* (Nonet *et al*, 1987; West and Corden, 1995). At the nonpermissive temperature (37°C), where the only source of Rpb1 is the thermostable S2A mutant, there was no indication of basal factor spreading (Figure 4D). Taken together, these results argue that CTD serine 2 phosphorylation does not regulate the dissociation of basal transcription factors from elongating RNApII.

Next, we asked whether the kinase activity of Ctk1 was required for the dissociation of basal transcription factors. Two mutant alleles were employed: *ctk1*-D324N is a catalytic site mutant that shows no kinase activity *in vitro* and confers a slow-growth, cold-sensitive phenotype *in vivo*, whereas *ctk1*-T338A is a weakened kinase mutated in the T-loop threonine that accepts an activating phosphorylation (Ostapenko and Solomon, 2003). Transformation of empty vector into a *ctk1*Δ strain produced no change in TBP cross-linking, which was still seen in the *PMA1* coding region (Figure 4E). Surprisingly, expression of the *ctk1* alleles with weak (T338A) or undetectable (D324N) kinase activity completely restored the normal pattern of TBP cross-linking. Therefore, it is the presence of Ctk1 itself, rather than its kinase activity, that confines basal transcription factors to the promoter.

Ctk1 is required for the stability of the scaffold

Hahn and colleagues have performed *in vitro* studies to identify factors that remain at the promoter after transcription initiation (Yudkovsky *et al*, 2000). They describe a complex, termed the 'scaffold', that appears primed for reinitiation. This complex contains the basal transcription factors TFIID, TFIIA, TFIIH, and TFIIE, Mediator complex, and an activator. As it is primarily the basal factors of the scaffold that exhibit aberrant *in vivo* cross-linking in cells lacking Ctk1 (Figure 1), we tested whether *in vitro* scaffold formation was affected in extracts from a *ctk1*Δ strain. In these assays, we would expect lower levels of scaffold factors to remain after a round of transcription if these basal factors inefficiently disengage from the escaping RNApII.

Preinitiation complexes were assembled on the HIS4 promoter linked with magnetic beads as described previously (Figure 5A) (Ranish *et al*, 1999; Yudkovsky *et al*, 2000). In the Hahn study, complexes were washed before initiation, and under these conditions, little or no Ctk1 associates with PICs (Liu *et al*, 2004). As *in vitro* experiments with mammalian Cdk9 (Wada *et al*, 1998; Chao and Price, 2001) and ChIP experiments with Ctk1 (Cho *et al*, 2001) also suggest that these kinases function after initiation, we modified the protocol. Where indicated, scaffolds were formed in extracts by the addition of ATP, or transcription was initiated by the addition of nucleotides for 2 min (Figure 5B). This time is only sufficient for a single round of transcription to occur (Ranish *et al*, 1999). After nucleotide addition, complexes were washed and proteins remaining on the templates characterized by immunoblotting (Figure 5C). Our results are in general agreement with those reported previously. In WT cell extracts, 62–95% of scaffold factors (TBP, TFIIH, and TFIIE) remain associated with the template, intermediate levels (23–42%) of RNApII Mediator components (Srb2 and Srb4), and little or no non-scaffold components (RNApII, TFIIB, and TFIIF) (Figure 5C and D).

Overall *in vitro* transcription was approximately equal between WT and *ctk1*Δ extracts (not shown), as was the recruitment of basal factors to the PIC before initiation (Figure 5C, lanes 2 and 6). In addition, the non-scaffold components RNApII, TFIIB, and TFIIF still dissociated efficiently from the promoter in the presence of NTPs or ATP alone. In contrast, upon scaffold formation in *ctk1*Δ extracts, we observed reduced amounts of TBP (WT to *ctk1*Δ: 75–40%), Tfb1 (TFIIH, 62–10%), Kin28 (TFIIH, 70–22%), and Tfa2 (TFIIE, 95–40%), whereas the level of Mediator components increased (Srb2, 42–78%; Srb4, 23–80%). Taken together, these results suggest that Ctk1 is required at an early post-initiation step to maintain scaffold stability. This finding is consistent with a model where Ctk1 regulates the dissociation of basal transcription factors from RNApII as it moves into productive elongation.

These immobilized template assays also allowed us to determine whether those basal transcription factors travelling with RNApII in Ctk1-deficient cells remain stably associated with polymerase after it runs off the template. A single round of transcription on the HIS4 template was allowed, and RNApII was immunoprecipitated from the supernatant through the Rpb3 subunit (Figure 5E). Although we can efficiently pull down Rpb3 itself, we failed to coimmunoprecipitate any of the basal transcription factors that cross-linked throughout genes in *ctk1*Δ cells (Figure 5F).

Discussion

The transition between transcription initiation and elongation requires a significant exchange of the factors associated with RNApII. The basal transcription factors and Mediator at the promoter must release the elongating RNApII. Elongation factors, such as the Spt4/5, TREX, and Paf complexes, subsequently associate with RNApII throughout the transcribed region (Kaplan *et al*, 2000; Pokholok *et al*, 2002). Although serine 2 phosphorylation of the Rpb1 CTD mediates the coupling of transcription with polyadenylation at 3′-ends (Ahn *et al*, 2004; Ni *et al*, 2004), our data suggest that Ctk1 also affects the transition from transcription initiation to elongation at 5′-ends of genes by promoting dissociation of basal factors from polymerase.

Surprisingly, this 5′ function of Ctk1 does not require kinase activity (Figure 4). Thus, it is most likely that Ctk1 has a structural function, either directly displacing basal factors or by recruiting elongation factors to the polymerase (perhaps through protein–protein contacts independent of phosphorylation) that subsequently mediate the dissociation of basal transcription factors from elongating RNApII. One possibility is that the Ctk1/Ctk2 and Kin28/Ccl1 CTD kinase/cyclin modules occupy the same physical space near the Rpb1 CTD, making their binding mutually exclusive. In the absence of Ctk1, contacts between RNApII and basal factors apparently persist during transcription elongation, resulting in the downstream cross-linking of basal factors (Figure 5G).

After initiation of transcription *in vitro*, the basal transcription factors TFIID, TFIIA, TFIIH, and TFIIE, as well as the activator and Mediator, remain at the promoter in the scaffold complex. This complex may facilitate subsequent rounds of

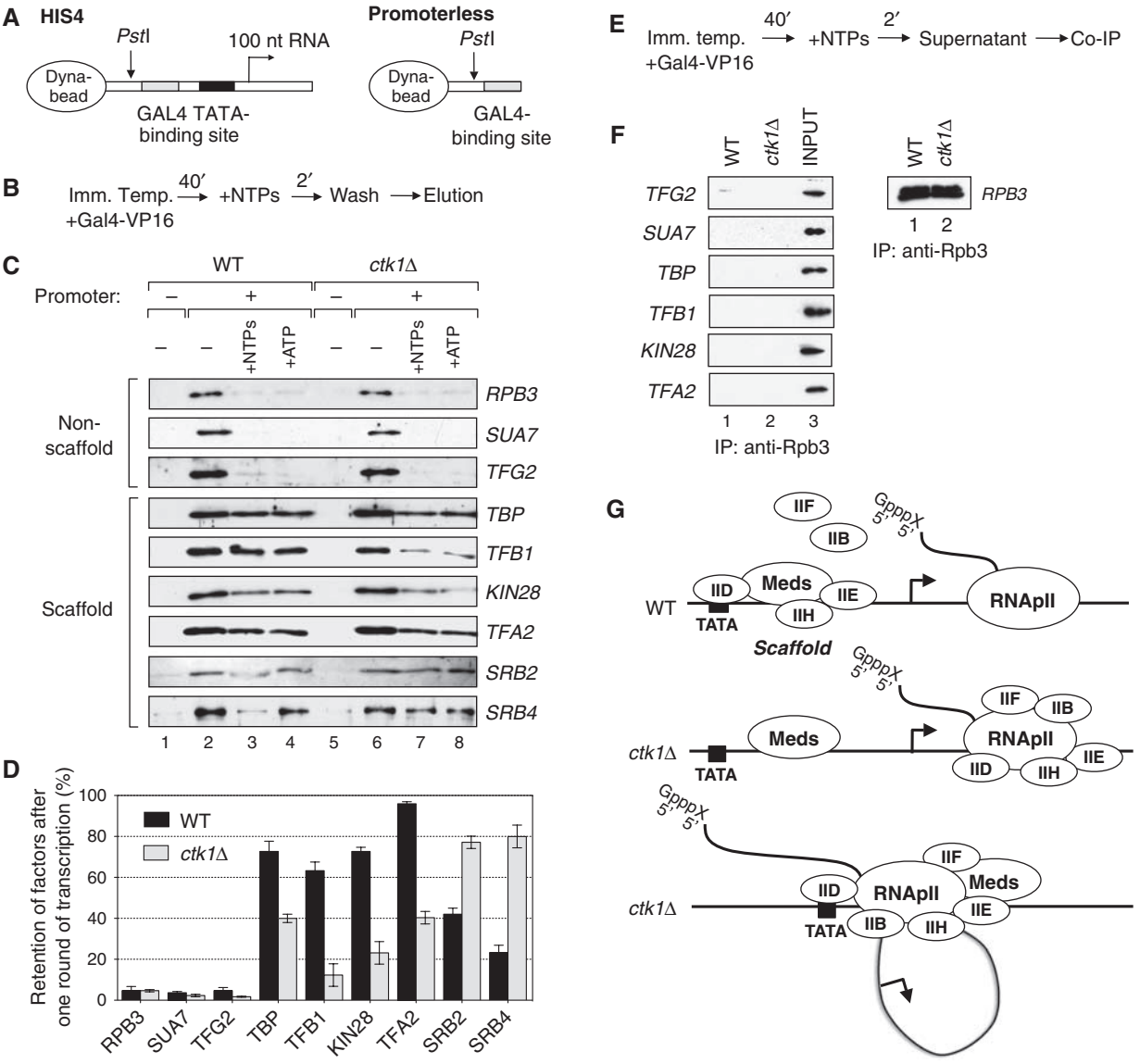

Figure 5 Ctk1 is required for the stability of the scaffold. (**A**) Immobilized templates used in this study. The HIS4 template contains a single Gal4 DNA-binding site upstream of the HIS4 core promoter containing a TATA box and transcription start site. The Promoterless template retains the Gal4 DNA-binding site but lacks the HIS4 promoter. Templates were immobilized on magnetic Dynabeads. (**B**) The scaffold formation assay. Cell extracts were incubated with the immobilized templates and activator Gal4-VP16 for 40 min. For single round measurement of transcription, PICs were incubated with NTPs for 2 min. (**C**) Immobilized templates were incubated with the activator Gal4-VP16 and yeast whole cell extracts from either WT (YSB726) or *ctk1*Δ (YSB854) for 40 min. After the formation of PICs, either nucleotides or ATP was added and incubated for 2 min. The templates were then washed and isolated by digestion with *Pst*I and detected by western blot. Lanes 1 and 5 show the controls for non-specific binding without promoter. Lanes 2 and 6 show typical PIC formation at the promoter. (**D**) Efficiency of scaffold formation was measured by quantitation of band intensities from lane 3 or 7 in WT and *ctk1*Δ cells (ImageJ v1.32). Signals from lane 2 or 6 were used as a control for each quantitation. Error bars are from three independent repetitions. (**E**) The scaffold assay after transcription termination. The cell extracts from either WT (YSB726) or *ctk1*Δ (YSB854) were incubated with the HIS4-immobilized templates and Gal4-VP16 for 40 min. To measure the association between RNApII and the basal transcription factors after termination, the supernatant was removed after incubation with NTPs for 2 min and immunoprecipitated with anti-Rpb3 antibody. (**F**) Coimmunoprecipitated proteins with Rpb3 from WT and *ctk1*Δ cells were determined by western blot using antibodies against non-scaffold (Tfg2 or Sua7) and scaffold components (TBP, Tfb1, Kin28, or Tfa2). (**G**) Model for the dissociation of basal transcription factors from elongating RNApII. See discussion for details.

transcription reinitiation (Yudkovsky *et al*, 2000; Hahn, 2004). Hahn and colleagues observed that the kinase activities of the TFIIH subunit Kin28 and the Mediator Subunit Srb10 contribute to the dissociation of polymerase, TFIIB, and TFIIF from scaffolds (Yudkovsky *et al*, 2000; Liu *et al*, 2004). Our results suggest that Ctk1 contributes to scaffold maintenance. In *ctk1*Δ extracts, both scaffold and non-scaffold factors dissociate from template DNA after a single round of transcription (Figure 5). This result is consistent with a model where Ctk1 promotes the release of basal transcription factors from transcribing RNApII as it enters productive elongation.

There are at least two possible variations of this model that could explain the ChIP results (Figure 5G). In the first, basal factors release from the promoter in *ctk1*Δ cells, but remain associated with the polymerase as it moves through the gene body. In the second, RNApII actually remains tethered to the basal factors at the promoter while transcribing. This would result in the transcribing polymerase 'pulling' the transcribed region past

basal factors, thereby allowing cross-linking with downstream sequences.

Although a function for Ctk1 in the 5′ transcriptional transition is a new finding, the presumed mammalian homologue Cdk9/P-TEFb has been shown previously to modulate early elongation (Chao and Price, 2001; Yamada *et al*, 2006). At least part of Cdk9 function at 5′-ends is mediated by its kinase activity, but it is certainly possible that there are additional structural functions. There are several steps before productive transcription elongation, including promoter proximal pausing, clearance, and escape (Goodrich and Tjian, 1994); further work will be required to determine exactly how Ctk1 and Cdk9 affect each of these.

Materials and methods

Plasmids and yeast strains

pY1A^2(8)WT(7): rpb1 (eight S2A mutant repeats, seven WT repeats) with C-terminal HA tag, *LEU2*, CEN/ARS, f1 + ori, Amp^R; pY1WT(10): rpb1 (10 WT repeats) with C-terminal HA tag, *LEU2*, CEN/ARS, f1 + ori, Amp^R; pY1A^2(14): rpb1 (14 S2A mutant repeats) with C-terminal HA tag, *LEU2*, CEN/ARS, f1 + ori, Amp^R; pRP114: *RPB1* (complete), *LEU2*, CEN/ARS, Amp^R; YCplac22-CTK1HA (T338A): ctk1 (T338A) with C-terminal HA tag (CTD kinase), *TRP1*, CEN/ARS, Amp^R; YCplac22-CTK1-DN: ctk1 (D324N) (catalytic domain mutant), *TRP1*, CEN/ARS, Amp^R; pJYC1513/pRS316-CTK1: *CTK1* (CTD kinase), *URA3*, CEN/ARS, f1 + ori, Amp^R. Strains used in this study are listed in Supplementary Table S1. Cells deficient in Ctk1 were deleted at the genomic locus but covered by pCTK1, a low-copy *URA3* plasmid containing WT Ctk1. Before our analyses, cells were grown overnight on YPD, streaked onto 5-FOA to select for those who lost the covering plasmid (confirmed by slow growth) and immediately used for subsequent experiments. We note that in some *ctk1*Δ strain backgrounds, the abnormal cross-linking of basal factors observed in our ChIP analyses was not as pronounced.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as described previously (Ahn *et al*, 2004). For precipitation of TAP-tagged proteins, 10 μl of rabbit IgG agarose (Sigma) was incubated with chromatin solution overnight at 4°C. For Rpb1 (BWG16 Covance), Sua7, TBP, Kin28, Tfb1, Tfa2, and Rap1 (y-300, Santa Cruz) immunoprecipitations, the antibodies were preincubated with protein A-sepharose CL-4B (Amersham) for 1 h at room temperature and then incubated with chromatin solution overnight at 4°C. For Rpb3 immunoprecipitation, antibody (1Y26, NeoClone) was preincubated with protein G-sepharose (Amersham). PCR amplification was performed with primer pairs as described (Ahn *et al*, 2004). PCR signals were quantitated by Fujix PhosphoImager. To control for amplification efficiency and label incorporation with different primers, the ratio of each gene-specific product to that of a non-transcribed region of chromosome V was calculated from the input sample signals. The signal for each specific gene primer in the immunoprecipitation was then divided by this ratio to convert the signal to normalized units. This value was divided by the immunoprecipitation signal of the non-transcribed control product to determine the fold enrichment of the ChIP over background signals. As this number is a ratio, a value of 1 represents 'no signal' (i.e., a signal equal to background).

Northern blotting

For northern blot analysis, total RNA was extracted from the indicated strains using the hot phenol method. Total RNA (25 μg) was resolved on a 1% agarose gel and transferred to nylon membrane. Blots were stained with methylene blue staining to confirm efficient transfer and equal loading. The membrane was probed with a 5′ (584–807) or 3′ (2018–2290) *PMA1* probe followed by autoradiography. The 5′-*PMA1* sequence was PCR-amplified from genomic DNA using primers: 5-AAG TCG TCC CAG GTG ATA TTT TGC A-3 (sense) and 5-AAC GAA AGT GTT GTC ACC GGT AGC-3 (antisense). The 3′-*PMA1* used primers 5-CTA TTA TTG ATG CTT TGA AGA CCT CCA G-3 (sense) and 5-TGC CCA AAA TAA TAG ACA TAC CCC ATA A-3 (antisense). Each PCR fragment was radiolabelled by random hexamer priming.

Immobilized template assay

Biotinylated templates were prepared by PCR as described previously, with pSH515 as a template (Ranish *et al*, 1999). Upstream p965 (5′-biotin-TAA TGC AGC TGG CAC GAC AGG-3′) and downstream pNot (5′-GGC CGC TCT AGC TGC ATT AAT G-3′) primers were used. For synthesis of the Promoterless template from pSH515, p965 was used with primer BKS7 (5′-TAC CGA GCT CGA ATT CGG AGG-3′). Dynabeads M-280 Streptavidin (Dynal) were concentrated with a magnetic particle concentrator (MPC) (Dynal) and washed twice in Buffer T (10 mM Tris (pH 7.5), 1 mM EDTA, 1 M NaCl). The beads were resuspended in Buffer T containing 0.003% NP-40 at 10 mg/ml. Dynabeads were then incubated with 8.6 ng biotinylated template per μg of bead in Buffer T for 30 min at room temperature with constant agitation. Immobilized templates were washed once in Buffer T and blocked in blocking buffer (1 ml/mg beads) for 15 min at room temperature. Blocking buffer consists of transcription buffer (10 mM HEPES (pH 7.6), 100 mM potassium glutamate, 10 mM magnesium acetate, 5 mM EGTA, 3.5% glycerol) containing 60 mg/ml casein, 5 mg/ml polyvinylpyrrolidone, and 2.5 mM DTT. The beads were concentrated with the MPC, washed three times in transcription buffer, and resuspended in transcription buffer to a final concentration of 10 mg/ml. After preparation of templates, the PIC assembly reaction was performed as described previously (Yudkovsky *et al*, 2000). Briefly, 150 μg of WT or *ctk1*Δ yeast whole cell extracts were added to reaction mix containing 12 mM phosphocreatine, 2.5 mM DTT, 400 ng creatine phosphokinase, 0.05% NP-40, and 1 × transcription buffer. After 10 min incubation at room temperature, the mix was spun at 9k r.p.m. for 2 min 4°C. The supernatant was transferred to a new tube on ice and Gal4-VP16, which had been preincubated with 2.5 μl of immobilized template for 10 min at room temperature, and 1 μg *Hae*III digested *Escherichia coli* DNA competitor were added. After 40 min incubation, scaffold isolation was performed with minor modifications. NTPs or ATP was directly added to the PICs at a final concentration of 100 μM for 2 min without washing to initiate and allow only a single-round transcription. The templates were then washed three times with wash buffer (1 × transcription buffer containing 0.05% NP-40 and 2.5 mM DTT), and isolated by digestion with 60 U *Pst*I for 30 min at 37°C. Proteins bound to the immobilized templates were analysed by SDS–PAGE and detected by western blot using Pierce ECL kits. Band intensities were determined using ImageJ v1.32 (a public domain Java image processing program inspired by NIH Image).

Coimmunoprecipitation experiments

Preinitiation complex assembly reaction was performed with 150 μg of yeast whole cell extracts from WT or *ctk1*Δ strains and the HIS4 template as described above. After 40 min incubation, scaffold isolation was carried out. NTPs were directly added to the reaction to a final concentration of 100 μM for 2 min without washing the PICs to initiate and allow only a single-round transcription. The supernatant was then removed and incubated with protein G-sepharose (Amersham) overnight at 4°C with anti-Rpb3 antibody in buffer E (20 mM HEPES (pH 8), 350 mM NaCl, 10% glycerol, 0.1% Tween 20). Beads were washed five times with buffer E and loaded onto SDS–PAGE gels together with 50 mg of input material. Proteins were analysed by western blotting using antibodies against Tfg2, Sua7, TBP, Tfb1, Kin28, and Tfa2.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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